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Relapses in early-stage follicular lymphoma frequently develop via a divergent evolution from their clonally related precursor cells

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ABSTRACT

Follicular lymphoma (FL) develops through a stepwise acquisition of cooperative genetic changes with t(14;18)(q32;q21)/*IGH*::*BCL2* occurring early at the pre-B stage of B-cell development. Patients with FL typically show an indolent clinical course, remitting and relapsing with the eventual development of resistance to treatments. Interestingly, the majority of transformed FL do not progress directly from FL but originate from their clonally related lymphoma precursor cells (CLP). To examine whether such divergent tumour evolution also underpins the relapses in patients with early-stage FL, we investigated by targeted next-generation sequencing 13 cases (stage I=9; stage II=4), who showed complete remission (mean: 5 years; range: 1–11.5 years) following local radiotherapy but subsequently relapsed (≥2 in 5). Clonal relationship between the diagnostic FL and relapses was confirmed in 11 cases. In 6 cases, common and distinct variants were seen between the paired diagnostic and relapsed lymphomas, indicating their divergent evolution from a CLP. In 2 cases, different B-cell clones were involved in the diagnostic and relapsed lymphomas, including 1 case involving 2 different *BCL2* translocations. In the remaining 5 cases, the relapsed lymphoma developed via a linear progression (n=4) or a mixed evolutionary path (n=1). These findings may bear important implications in the routine diagnosis and management of relapsed FL.

Key words: Follicular lymphoma, lymphoma recurrence, mutation profiling, tumour evolution

INTRODUCTION

Follicular lymphoma (FL) is an indolent B-cell lymphoma, characterised by a spectrum of somatic genetic changes, including t(14;18)(q32;q21)/*IGH*::*BCL2*, which occurs early at the pre-B stage of B-cell development in the bone marrow as a result of erroneous VDJ recombination. The majority of patients with FL undergo an indolent clinical course, remitting and relapsing, and eventually developing resistance to standard therapies, while ~30% of cases may show transformation into a more aggressive lymphoma, often diffuse large B-cell lymphoma (DLBCL), also known as transformed FL (tFL) [1]. Remarkably, the majority of tFL do not progress directly from FL but originate from their clonally-related lymphoma precursor cells (CLP) [2–4]. Most cases investigated in the literature are paired FL and transformed FL, and do not have detailed information on clinical stage, or treatments and their responses, particularly the complete remission (CR) status. In the present study, we investigated the evolution of lymphoma relapse in a series of well-documented patients with early-stage FL, who had achieved CR following local radiotherapy. As there is no systemic therapy involved, and thus no therapeutic effect on CLP cells, these cases provide a unique opportunity to study the natural history of the CLP cells, greatly complementing the previous studies on this topic.

MATERIALS AND METHODS

*[EdQ: please provide **city**, **state or province**, **country** details for each supplier of equipment or reagents or **software** at first mention; thereafter use only the supplier name. Please repeat this process in supplementary items as well as in the main text.]*

Case materials: The use of archival tissues for research was approved by the ethics committees of the institutions involved. A total of 13 patients with early-stage FL (9 stage-I; 4 stage-II) were successfully investigated, and all achieved CR (mean: 6 years; range: 1–11.5

years) following local radiotherapy, but subsequently relapsed (Figure 1). Seven cases had 1 lymphoma relapse, while the remaining 6 cases had 2–5 relapses. In 7 cases, the relapsed lymphoma was FL, while in the remaining cases, the relapsed lesions were tFL (n=2), or both FL and tFL (n=4) including an EBV-positive LBCL (case 11). Paired formalin-fixed paraffin-embedded diagnostic and relapsed lymphoma biopsies were available in each case.

Interphase fluorescence *in situ* hybridization (FISH): This was performed to investigate *BCL2* translocation using *BCL2* Break Apart probes (Vysis, Abbott Park, IL, USA).

DNA extraction and quality assessment

Histology was reviewed and areas rich in lymphoma cells (>30%) in each specimen were microdissected, and subsequently subjected to DNA extraction and a quality control PCR [5].

Clonality analysis of *IG* gene rearrangements: This was performed by combining the BIOMED-2 PCR assays and Illumina MiSeq sequencing [Illumina, Inc., San Diego, CA, USA] [6].

Targeted next-generation sequencing (NGS)

A customised panel of 191 genes for FL and DLBCL was used for mutational analyses. Targeted NGS was performed with 100 ng DNA using TWIST capture [TWIST Bioscience, San Francisco, CA, USA] and the Illumina NextSeq platform. In 7 specimens with suboptimal DNA quality, the targeted sequencing was performed in duplicates. The gene panel and NGS methodology were detailed in the supplementary materials [5–7].

RESULTS AND DISCUSSION

Interphase FISH showed *BCL2* translocation in 6 cases, *BCL2* amplification (up to 8 copies) in 1 case, and *BCL2* copy number gain (3–4 copies) in 3 cases. In 3 cases, both diagnostic and relapsed lymphomas were investigated by interphase FISH, with identical or similar results between the paired samples.

Adequate sequencing coverage (>98% with a minimum of 100 reads after deduplication) for all the targeted genes was achieved in each specimen investigated. The variants detected were typical of those seen in FL and DLBCL. There was no significant difference in the number of potentially pathogenic mutations detected between the diagnostic (mean: 7.8/sample; range: 1–13) and relapsed FL (mean: 7.9/sample; range: 2–13) excluding tFL. Both diagnostic and relapsed FL (excluding tFL) showed similar mutation frequencies in *BCL2*, *CREBBP*, *EP300*, *KMT2D*, *TNFRSF14* and *STAT6* (supplementary material, Figure S1). There was no association between *STAT6* mutation and *BCL2* translocation status or CD23 expression by immunohistochemistry.

To investigate the clonal evolution of the diagnostic and relapsed lymphomas, we compared all somatic variants in the paired samples, including pathogenic, benign, and synonymous variants, and variants in *BCL2* 5'- and 3'-UTR. The clonal relationship between the diagnostic FL and relapses was confirmed in 11 cases by their shared somatic variants.

In 6 cases, both common and distinct variants were seen between the paired diagnostic and relapsed lymphomas, indicating divergent evolution of these lesions from a CLP (Figure 2). The malignant status of the inferred CLP cells is uncertain. *In situ* follicular B-cell neoplasia (ISFN), a

premalignant lesion, may harbour variable numbers (0–4) and combinations of pathogenic mutations [6,8,9]. In 4 of these cases (no. 1, 3, 5 & 6), the inferred CLP cells only harboured 1–3 potentially pathogenic mutations (excluding benign changes), and are thus probably pre-malignant. In the remaining 2 cases (no. 2 & 4), the inferred CLP cells harboured ≥7 potentially pathogenic mutations in addition to *IGH*::*BCL2*, and they are likely malignant or in the phase of malignant transformation.

In two cases, there was evidence of two B-cell clones that gave rise to multiple lymphomas independently. In one case (no. 7), different *IGH* rearrangements were seen between FL-D and tFL-R2 (Figure 3). There were also no shared somatic variants between the diagnostic FL and the two "relapsed" lesions, in keeping with their derivation from different B-cell clones. In the other case (no. 8), interphase FISH with the *BCL2* breakapart probe demonstrated a *BCL2* translocation in each of these lymphomas, but displayed different constellation patterns. FL-D and FL-R1 showed evidence of an unbalanced *BCL2* translocation (1–2 co-localised red/green & 1 red signal), while FL-R2 and FL-R3 displayed a classic breakage pattern (1 co-localised red/green, 1 red and 1 green signal) (Figure 3). Immunohistochemistry showed strong and uniform BCL2 expression in the lymphoma cells of these specimens. PCR for *IGH*::*BCL2* genomic fusion did not yield any products within the expected size range in each of these specimens. Nonetheless, clonality analyses displayed distinct sized FR2-JH products between FL-R1 and FL-R2, indicating different *IGH* rearrangements, and this was further confirmed by sequencing these PCR products (Figure 3). In line with these findings, FL-D and FL-R1 shared 1 *CREBBP* mutation, while FL-R2 and FL-R3 had 4 common mutations. However, there were no common variants between FL-D/FL-R1 and FL-R2/FL-R3. Taken together, these findings

indicate that FL-D/FL-R1 and FL-R2/FL-R3 originated from two different B-cell clones, which also harboured different *BCL2* translocations.

In 4 cases (no. 9–12), mutations were largely cumulative in consecutive lesions, suggesting linear progression of the relapsed lymphoma (Figure 4). In each of these cases, there were variable numbers (1–15) of shared somatic variants between the diagnostic and relapsed lymphomas. Although more genetic changes might be demonstrated by more extensive sequencing beyond the 191 FL/DLBCL associated genes investigated, this is unlikely to alter the conclusion of linear progression in these relapsed lesions. In the remaining case (no. 13), the relapsed lymphoma appeared first to follow a linear progression, then a divergent evolutionary path (Figure 4).

As many of the genes mutated in ISFN are affected by the off-target activities of the somatic hypermutation machinery and may confer no or moderate oncogenic activities, there exists a considerable challenge in stratifying the risk posed by these mutations and their combinations in lymphoma development [10,11]. Nonetheless, *CREBBP* mutations within the KAT domain have been found preferentially in the lymphoma precursor cells that eventually develop into overt FL [12]. In line with this, the majority of *CREBBP* mutations found the present study are within the KAT domain, seen in 9 cases including in the inferred CLP cells in 2 of the 8 cases where divergent evolution enables CLP prediction (supplementary material, Figure S2).

The above findings raise critical clinical implications in routine diagnosis and management of recurrent lymphoma in patients with FL. In routine clinical follow-up of patients with FL post-

therapy, re-biopsy is usually not considered unless a high-grade transformation is suspected. Even when a re-biopsy is performed, the routine diagnostic workup only includes *IG* gene clonality and *BCL2* translocation analyses, but not mutation profiling. Importantly, neither *IG* gene clonality nor *BCL2* translocation analyses can address the evolutionary path of the relapsed lymphoma, despite providing evidence about clonal relationship to the original FL.

As the majority of relapsed lymphomas (both FL and DLBCL) in patients with FL originate from their clonally related CLP cells via a divergent evolution or a different B-cell clone, these relapsed lymphomas are in fact a new tumour, rather than a true relapse. As new lymphomas, they responded to the same or similar treatments without the need for escalating the therapy, unless they were high-grade tumours. In this context, it is important to ascertain whether a recurrent lymphoma is a new tumour or a true relapse.

The above findings, along with the evidence of divergent evolution in the majority of tFL, challenge the current therapeutic strategy of FL because the *IGH*::*BCL2* positive premalignant cells largely underpin the lymphoma recurrence and their low proliferation and indolent nature most likely render them poorly responsive to immunochemotherapy designed for FL. Studies of ISFN have shown that *IGH*::*BCL2* positive premalignant cells can actively transit in peripheral lymphoid tissues, undergo relentless clonal expansion through germinal centre reactions while at risk of acquiring genetic changes due to exposure to somatic hypermutation and switch recombination activities, and also apoptosis evasion by BCL2 over-expression [6,8,9,13]. This insidious clonal evolution is formidable, inevitably propelling independent malignant transformation and hence the development of a new lymphoma. This is likely to explain why

FL is incurable based on the current therapeutic strategies despite being a low-grade tumour, and also highlights the need to control the *IGH*::*BCL2* positive premalignant cell population in order to achieve a cure.

In summary, the recurrent lymphomas in patients with early-stage FL frequently develop via a divergent evolution from their clonally related CLP cells or a different B-cell clone, further underscoring the potential of these premalignant cells in multi-lymphoma development.

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Author contributions statement [EdQ: I have rearranged – please check changes – perfect, thank you!]

JM, MMT, ZC, SG, DJ, CC, FC and MQD were responsible for experimental design, data collection and analysis. AW contributed cases and to assessment of pathology. JM, AW, MMT and MQD prepared and wrote the manuscript. AW and MQD designed and coordinated the study. All authors commented on the manuscript and approved its submission for publication.

Data availability statement: All laboratory data are presented in the figures and supplementary figures of the manuscript.

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FIGURE LEGENDS

Figure 1. Summary of cases with early stage follicular lymphoma (FL) and clinical follow up data. Complete remission (CR) was achieved following each treatment unless indicated (nCR: non-complete remission). tFL: transformed FL; RT: radiotherapy; CT: chemotherapy; WW: watch-and wait.

Figure 2. Cases showing divergent evolution in FL relapse.
FL: follicular lymphoma with grade indicated, D: diagnostic biopsy; CR: complete remission; R: relapsed lesions; tFL: transformed FL; CLP: predicted clonally related lymphoma precursor cells, and their mutations common to both diagnostic and recurrent lymphoma are indicated; SHM: somatic hypermutation; variants in blue are potentially pathogenic, while variants in green are synonymous or benign changes or present in UTR.

Figure 3. Cases showing involvement of different B-cell clones in FL relapse.
In case 7, the FL at diagnosis and recurrent FL (FL-R1 and tFL-R2) do not harbour common mutations and also bear different *IGH* gene rearrangement, thus are derived from different B-cell clones. In case 8, FL-D/FL-R1 and FL-R2/FL-R3 originate from two different B-cell clones as shown by their different mutation pattern, *BCL2* breakapart FISH signal constellation and *IGH* rearrangements. Arrows in the FISH panel indicate breakage of the green and red probe signals. FL: follicular lymphoma, D: diagnostic biopsy; CR: complete remission; R: relapsed lesions; CLP: predicted clonally related lymphoma precursor cells and their mutations common to both diagnostic and recurrent lymphoma are indicated; SHM: somatic hypermutation; variants in blue are potentially pathogenic, while variants in green are synonymous or benign changes or present in UTR.

Figure 4. Cases showing a linear progression or a mixed evolutionary path in FL relapse. FL: follicular lymphoma with grade indicated; D: diagnostic biopsy; CR: complete remission; R: relapsed lesion; tFL: transformed FL; ILC: predicted intermediate lymphoma cells; SHM: somatic hypermutation; variants in blue are potentially pathogenic, while variants in green are synonymous or benign changes or in UTR. Case 9 shows no further mutations in the recurrent lymphoma, however, this does not exclude any potential alterations in other genetic and epigenetic changes not investigated in the present study.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Figure S1. Mutation frequencies between diagnostic and relapsed FL excluding transformed FL.

Figure S2: Distribution of *CREBBP* mutations in diagnostic FL and relapsed lesions.

Table S1. The gene panel investigated by targeted next generation sequencing

Table S2. List of primers used